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Polymorphic microsatellite DNA markers in the penduline tit, *Remiz pendulinus*

L. A. MÉSZÁROS,* N. FRAUENFELDER,† M. VAN DER VELDE,‡ J. KOMDEUR‡ and J. SZABAD*
*Department of Biology, Faculty of Medicine, University of Szeged, Hungary, †Ecogenics GmbH, Zurich-Schlieren, Switzerland, ‡Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, The Netherlands

Abstract

To describe the exceptional mating system of the penduline tit, *Remiz pendulinus*, we aim to combine field observation records with DNA analysis based on polymorphic microsatellite DNA markers. Here we describe features of nine loci and their corresponding polymerase chain reaction primers. The observed number of alleles varied from four to seven and the observed heterozygosity ranged from 0.419 to 0.802. Neither of the loci is sex-linked and as linkage disequilibrium analysis showed they assort independently. Seven of the nine loci were polymorphic in the Cape penduline tit, *Anthoscopus minutus*.

Keywords: microsatellite, penduline tit, polymorphism, *Remiz pendulinus*

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The penduline tit, *Remiz pendulinus*, is a small Eurasian passerine bird with a curious mating system. During the breeding season (April–August), males may build as many as seven nests and have up to seven different mates. The females may join as many as four nest-owner males (Persson & Öhrström 1989). Following the formation of breeding pairs and laying of about four eggs per nest, the fate of the clutch follows one of three possibilities. (i) In 49% of the nests, the female alone takes care of the clutch and the offspring. The male departs and starts to build a new nest to attract a new female. (ii) In 11% of the cases while the female departs and seeks for a new mate, the male cares for the clutch. (iii) Rather unusually, in the remaining 40% of the nests, the clutches are abandoned by both parents (Szentirmai 2005). To resolve the rather unusual reproductive behaviour of the penduline tit an individual genetic fingerprint of every bird was determined in the Fehér-tó (White Lake) region, North of Szeged, Hungary, based on their polymorphic microsatellite DNA marker composition and kinship of this breeding population was established.

To identify penduline tit specific polymorphic microsatellite DNA markers, we first isolated genomic DNA from blood samples of 10 penduline tits. The blood samples were stored in Queen's buffer (Seutin *et al*. 1991) and DNA was isolated using the phenol-chloroform-isoamyl alcohol method (Krokene *et al*. 1996). Primer development was carried out at ecogenics GmbH, starting with a pool of 10 penduline tit DNA samples. An enriched library was prepared from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour *et al*. 1994) and enriched by magnetic bead selection with biotin-labelled $(CT)_{13}$ $(GT)_{13}$ $(GTAT)_7$ and $(GATA)_7$ oligonucleotide repeats (Gautschi *et al*. 2000a, b). Of the 374 recombinant colonies screened, 113 (69 GT/CT and 44 GTAT/GATA) gave a positive signal after hybridization. Plasmids from 51 positive clones were sequenced and primers were designed for 18 microsatellite inserts, all of which were tested for polymorphism in a sample of 10 supposedly unrelated adult penduline tits captured in the research area mentioned above. Of the 18 markers nine yielded clear and variable sized amplification products with four to five alleles each. Further alleles were identified when many more DNA samples were analysed. Features of the microsatellite markers and their corresponding PCR primers are summarized in Table 1.

Forward primers of the nine primer pairs were fluorescently labelled with either 6-FAM (blue), HEX (green) or NED (yellow). For PCR amplification, the conditions were as follows: each 10 μL reaction mixture contained 10–50 ng genomic DNA, 0.5 μM of each primer, 0.2 mM of each dNTP, 0.05 U of *Taq* polymerase (Advanced Biotechnologies) and 1.5 mM $MgCl_2$ in the prefabricated reaction buffer (final concentration 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl, pH 9.0,

Correspondence: János Szabad, Fax: +36 62 545109; E-mail: szabad@mdbio.szote.u-szeged.hu

Table 1 Characteristics of nine polymorphic microsatellite loci in the penduline tit *Remiz pendulinus*

Locus	EMBL Accession no.	Repeat motif	PCR primer nucleotide sequence (5′–3′)	Alleles	Allele size (bp)	Heterozygosity		
						Observed§	Expected§	Bird
PCR multiplex set 1								
Remiz-05	AM709789	$(AC)_{11}AT(AC)_4$	F: GATCCCGGTGATGCTCTTC‡ R: TCAACCAACTCCTTCCATCC	4	123, 125, 127, 129	0.740	0.638	550
Remiz-07	AM709790	$(CTAT)_{12}$	F: GGTAAGCTGGTGCACAAAATG† R: GGTCTATGAAAGATGATAGATGATGG	4	164, 168, 172, 176	0.693	0.664	550
Remiz-09	AM709791	$(CTAT)_4$ $TAT(CTAT)_{11}$	F: AATTACTGAAGAACAACACATCTGG* R: GGACAGCTGGAGAGCAACTC	5	114, 118, 122, 126, 130	0.419	0.712	554
Remiz-10	AM709792	$(CTAT)_{12}$	F: ATCACTCCCCAGTGATAGCC† R: CCTTCAGCACTGAGAATAGGG	6	196, 200, 204, 208, 212, 216	0.728	0.761	534
PCR multiplex set 2								
Remiz-01	AM709788	$(GATA)_{11}$	F: TGCCTTCTATCAAGCATGAGC‡ R: TGTGCATGTAAGATTTCCATCTATC	5	170, 174, 178, 182, 186	0.690	0.665	532
Remiz-11	AM709793	$(GT)_{14}$	F: TGCACTAATTGCCCAGTTTTC† R: AATGCTCCATTCATCATCTGC	5	120, 122, 124, 126, 128	0.718	0.682	550
Remiz-14	AM709794	$(AC)_{16}(GT)_3$	F: CTTCTGCTTGCCTTTTGAAAC* R: AACGATTTGAAATATGACTGC	6	207, 209, 217, 219, 221, 223	0.744	0.772	536
PCR multiplex set 3								
Remiz-17	AM709795	$(CTAT)_{13}$	F: CCTATCTGTCCATAGCCTTCTCTAC* R:GGATGAGAAAGTTCATGTTTTATGG	5	144, 148, 152, 156, 160	0.721	0.705	535
Remiz-18	AM709796	$(GATA)_{10}$ $(GACA)_2(GATA)$	F: CATTAATGATTGGATATGGCAAG† R: GTCCCTCTGCCTGTCGTTC	7	86, 90, 94, 98, 102, 106, 110	0.802	0.810	535

The annealing temperature was 56 °C in every PCR.
*6-FAM-labelled.
†HEX-labelled.
‡NED-labelled.
§Based on Hardy–Weinberg equilibrium analysis.

0.01% (w/v) Tween). Polymerase chain reaction (PCR) amplifications were carried out in a Thermolyne amplitron II or in a Corbett Research thermocycler device. The PCR amplifications started with one denaturation cycle at 94 °C for 3 min, followed by 40 cycles at 94 °C for 30 s, 56 °C for 60 s, 72 °C for 30 s and a final extension step at 72 °C for 10 min. The nine microsatellite loci were amplified in three multiplex sets: (i) Remiz-05, Remiz-07, Remiz-09 and Remiz-10, (ii) Remiz-01, Remiz-11 and Remiz-14, and (iii) Remiz-17 and Remiz-18. Because loci with overlapping size ranges were distinguished by the use of primers labelled with different dyes, the multiplex sets 2 and 3 could be mixed after PCR to reduce the number of samples to be analysed by gel electrophoresis. One microlitre of each sample was mixed with 1.5 μL loading buffer that contained 1.1 μL deionized formamide, 0.18 μL blue dextran loading dye and 0.22 μL internal size standards (ROX350, Applied Biosystems). The samples were then denatured at 94 °C for 2–3 min and placed on ice right away. 1–1.2 μL were loaded on a 10% denaturing polyacrylamide gel in an Applied Biosystems (ABI) 377 XL DNA sequencer. Allele calling and sizing were performed using the GENESCAN 3.1 (Applied Biosystems) and the GENOTYPER 2.5 software.

All nine loci were tested for Hardy–Weinberg equilibrium using the CERVUS 3.0 software (Kalinowski *et al*. 2006). The expected heterozygosity ranged from 0.638 to 0.810, and the observed heterozygosity from 0.419 to 0.802 (Table 1). While eight loci displayed no significant deviations from the Hardy–Weinberg equilibrium, heterozygote deficiency was apparent in one of the loci (Remiz-09). None of the nine loci is sex-linked and neither of the alleles is null according to the MICRO-CHECKER 2.2.3 software (Van Oosterhout *et al*. 2004). Linkage disequilibrium analysis of the nine loci was carried out by the GENEPOP 3.4 software (Raymond & Rousset 1995; http://genepop.curtin.edu.au). The analysis (based on 302 birds in 50 families) clearly showed that the nine loci assort independently as shown by the corresponding values that ranged between 0.948 and 1.000.

We have been successfully using the nine polymorphic markers in DNA-based identification of about 1200 penduline tits in order to establish kinship and elucidate the unusual mating system.

Table 2 Features of the nine polymorphic loci in 10 Cape penduline tit *Anthoscopus minutus*

Locus	No. of alleles	Allele size s (bp)
Remiz-05	—	No PCR product
Remiz-07	2	178, 182
Remiz-09	4	126, 130, 134, 138
Remiz-10	2	208, 212
Remiz-01	2	170, 174
Remiz-11	1	109
Remiz-14	2	185, 187
Remiz-17	2	186, 206
Remiz-18	3	86, 102, 112

We also tested the nine microsatellite loci on 10 Cape penduline tit (*Anthoscopus minutus*) DNA with the following results: while one of the loci could not be amplified and one showed no sign of polymorphism, seven of the nine markers are polymorphic (Table 2). Perhaps a higher degree of polymorphism would appear upon analysis of a larger DNA sample size and may thus make kinship analysis feasible in the Cape penduline tit.

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